MIGRATORY VARIATIONS IN GLIOBLASTOMA SUBTYPES

UNDERGRADUATE HONORS THESIS

Presented in partial fulfillment of the requirements for a Bachelor of Science with Honors Research Distinction in Biomedical Engineering from The Ohio State University

By

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ABSTRACT

The prognosis for glioblastoma multiforme (GBM) is currently very grim; median survival is 14.6 months for this high grade brain cancer. Complete eradication of GBM is very unlikely, and it is not curable with current chemotherapy or surgical treatment options. GBM's reoccurrence is strongly related to its ability to infiltrate and migrate to other areas of the brain, as far as the opposite hemisphere. There is increasing evidence supporting the initiation of GBM through glioma stem cells. Recent evidence suggests that GBM has three subtypes with different genetic expression signatures. Even though prognosis is consistent, the responses to aggressive treatment differ between the subtypes. This suggests that identifying the predominant subtype between patients may allow for stronger, more individualized treatments. We hypothesize that these distinct genetic signatures will lead to different migration patterns through activation of different chemical pathways, which may serve as therapeutic targets. To investigate this, electrospun nanofiber models, which mimic white matter tracts, major migratory tracts for GBM invasion in the brain, were used to compare migration patterns between two, patient derived subtypes: "Proneural" and "Mesenchymal." Confocal microscopy indicated that there is a clear distinction in cell adhesion and morphology, while time-lapse microscopy showed a statistical variance in and migration speed. Preliminary data suggests a potential clinical relevance in the treatment and prognosis of different types of GBM, particularly if the specific molecular pathways involved can be identified. Elucidating how individual molecular pathways affect subtype function is crucial in the

treatment and understanding of these glioma stem cells. This system will ultimately function as a platform to delve deeper into the understanding of subtype migration and further analysis for targeted treatments

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FIELDS OF STUDY

Major Field: Biomedical Engineering, Pre-medicine

Minor Field: Entrepreneurship

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1. Introduction

Glioblastoma multiforme (GBM) is the most common form of malignant brain tumors in adults[i], affecting approximately 22,500 individuals annually in the United States. Those diagnosed with GBM have a low median survival rate of ~15 months and a five-year survival rate of ~4%.[ii] Since prognosis is grim, there is a large need to better understand the complex and unique behavior of GBM tumors. The Cancer Genome Atlas (TCGA), a national effort to map the genomes and catalogue the genetic mutations of cancer types, selected GBM as the first brain tumor for its project. Since then, genomic subclassifications of GBM have been identified.[iii] These GBM subtypes have been studied for their clinical significance and, for example, patients with Mesenchymal GBM have shown to respond to aggressive treatments differently when compared to other subtypes of GBM.[iv] However, all subtypes of GBM still have similar median survival rates. This indicates that more information is needed on the phenotypic behavior of each individual subtype in order to more effectively treat GBM according to its individual subtype.

Subtypes names were assigned based on expression of signature genes. Mesenchymal subtype expresses CHI3L1 and MET, markers described by Phillips et al. as being mesenchymal markers.[v] In addition, the combination of upregulating MERTK and CD44 is a familiar pattern that has been associated with the epithelial-to-mesenchymal transition and de-differentiated tumors.[vi] The Mesenchymal subtype is also associated with low expression of NF1,[vii] a negative regulator of ras, which is a protein involved in

adhesion and cell migration. Low expression of NF1 leads to poor adhesive and migratory behavior from the Mesenchymal subtype.

The Proneural subtype was mainly associated with point mutations in IDH1 and amplifications in PDGFRA. In addition, high expressions of PDGFRA, NKX2-2, and OLIG2, which have been shown in the Proneural subtype, are considered to be oligodendrocytic development genes.[viii] Over expression of OLIG2 has been shown to increase proliferation.[ix] Several proneural development genes, such as SOX genes, DCX, DLL3, ASCL1, and TCF4, were shown in the Proneural subtype,[v above] explaining the naming of the subtype.

According to Verhaak et al., glioblastoma subtypes are similar to distinct neural cell types. The signature of Mesenchymal subtype correlated strongly with cultured astroglial signatures. Proneural cells, on the other hand, showed high similarities to oligodendrocytic signatures.[iv] Phenotypically, in in vitro conditions where cells were grown in suspension, Proneural cells were shown to form neurospheres, while Mesenchymal floating aggregates where loosely formed and irregularly-shaped.[xiv] This is consistent with mesenchymal cell in vivo, where low cell-to-cell adhesion is shown.[x]

To investigate the phenotypic differences between subtypes, migratory, adhesive, and morphological behavior between Proneural and Mesenchymal subtypes were compared. Conclusions from this study confirm that there are differences in the behavior between the subtypes and suggest that there could be therapeutically relevant chemical pathways that could be identified through gene sequencing the GBM cell lines from the different subtypes. Further investigation would better characterize the phenotypic behavior each individual subtype.

Our studies were conducted on highly aligned nanofiber models. Previous studies have shown that highly-aligned electrospun nanofibers closely mimic white matter tracts in the brain.[xi] White matter tracts are a major migratory pathway used by GBM cells.[xii] Therefore, the aligned nanofiber model is the optimal model that can be used for an in vitro study on GBM migration.

The study on migratory variations between GBM subtypes is significant because it provides a clear pathway for future works to be done on the specialization of treatment for different GBM subtypes. Currently, the prognosis for GBM patients is consistently unfavorable among all subtypes, with median survival time slightly over a year. However, with a better understanding of what specific behaviors are inconsistent amongst the GBM subtypes, clearer indications will be given on which chemical pathways are therapeutically relevant.

The existence of GBM subtypes is known, but it is not known how knowledge of these subtypes can be translated into more effective treatment for patients. Therefore, it is imperative that investigative research be done. Nanofiber models are optimal method to gather quantitative data on migratory behavior of each subtype since it has been proven that they closely mimic in vivo conditions in the brain. This approach could potentially be used to study a variety of metastatic cancers, in addition to GBM. Nanofiber models represent a practical way to obtain this data.

When considering clinically relevant pathways, some inhibitors already exist for components of these pathways. These inhibitors could be used as possible therapeutics for patients with the associated subtype of GBM. An example of this would be that

epidermal growth factor receptor (EGFR) is upregulated in "Mesenchymal" GBM, so an existing inhibitory drug for EGFR could be used as a treatment option.

The goal of this study is to contribute to the knowledge of the differences in behavior between differing subtypes of GBM. Procedures testing the variances in migration, adhesion, and morphology between Proneural and Mesenchymal subtypes will be quantified using highly-aligned electrospun nanofiber models. Analysis of the data has initially indicated that there are statistically relevant differences in all categories between the two subtypes.

These conclusions indicate that there are additional relevant avenues to investigate that would lead to further understanding in the differences between the two subtypes. Moving forward in the study, additional cell lines should be examined for each GBM subtype to ensure that migration behavior for each line are characteristic of behavior for all cell lines in its subtype. A specific aim of the study would be to determine if migration, adhesion, and morphology of GBM subtypes will differ on nanofiber models. Different genetic signatures will lead to different migration patterns, and possibly different mechanisms of cancer invasion.

An additional aim of the study will be to analyze protein expression associated with migration (e.g., FAK, MLCII, EGFR, and/or vimentin). Protein expression will presumably differ between cell types, which can be compared through western blotting. Protein expression will then be compared to our genetic signatures for each cell type. Protein expression from western blots and gene expression from our gene maps will be evaluated. This will make suggestions for potential patient stratification and personalized drug therapies.

After more diverse sets of data are gathered, the results should be compared with gene sequence maps for each cell line. Inconsistencies in the gene maps between the two subtypes should be investigated in order to determine chemical pathways that could be interesting therapeutic targets. With the end goal of this study to identify targets for therapeutic research, it is possible that advances of knowledge in the migration behavior of GBM subtypes could lead for more comprehensive treatment of GBM, and a more positive prognosis for GBM patients.

2. Materials and Methods

2.1 Preparation of aligned PCL nanofibers

2.1.1 Electrospinning

Nanofibers were made using procedures outlined by Rao, et al. In short, 5 wt% PCL (Mn 70,000–90,000, Sigma–Aldrich, St. Louis, MO) was prepared in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (>99% purity; Oakwood Products, Inc., Columbia, SC) and poured into a 20 cc syringe fitted with a 20 gauge blunt tipped needle connected to a nozzle (Small Parts Inc., Miramar, FL). The syringe was set to a flow rate of 5 mL/h and electrospun using a DC high voltage power supply (Glassman High Voltage, Inc., High Bridge, NJ) at positive 20 kV. The needle-to-collector distance was to 20 cm and electrospun for ~ 25 minutes at an average relative humidity of 30%. Fibers were deposited on tissue culture polystyrene (TCPS) substrates rotating at a linear velocity of 15 m/s to produce to aligned fibers.[xiii]

Plasma treatment was done in order to stay consistent with prior testing.[xiii] Nanofibers were placed into a Harrick plasma cleaner chamber (Harrick Plasma, Ithaca, NY, USA) under vacuum at 1000 mTorr and exposed to plasma radio frequency of 8-12 MHz for 3 minutes. Fibers were protected from light and moisture while in storage.

2.1.2 Sterilizing Nanofiber Scaffolds

A 16 mm metal punch (Arch Punch; C.S. Osborne & Co, Harrison, N.J.) was used to cut fibers when ready for cell culture experiments. Fiber disks were fixed to the bottom of a 12 well plate using medical adhesive (Dow Corning Silastic Brand, Medical Adheisve,

Silicone Type A). Glue was allowed to dry. For sterilization, fibers were incubated at 37°C in 70% ethanol under UV light for 45 minutes. Fibers were then washed with sterile phosphate buffer saline (PBS) (3x) and allowed to dry overnight.

2.2 Patient derived GBM cell culture

Cells used for this study were borrowed from Nakano et al. and were derived under a previously established protocol.[xiv] In short, specimens were collected from 40 patients with high-grade gliomas and 19 viable cell lines were established using a defined serum-free media that promotes proliferation and supports multipotent glioma cancer stem cells. Culture conditions gave rise to two phenotypically distinct glioma stem cells (n=10 and n=9). Immunocytochemistry confirmed stem-cell associated markers were present. Group 1 was highly positive for Sox2, a marker for Proneural gliomas. Group 2 was positive for CD44 but negative for Sox2, consistent with Mesenchymal glioma stem cells.

Groups 1 and 2 were characterized into Proneural and Mesenchymal based on mRNA expression by Nakano et al. A differential expression analysis of Proneural vs Mesenchymal was performed and 5,796 genes were found to be differentially expressed. One group's expression was consistent with the Proneural subtype, and the other with Mesenchymal. Furthermore, 1,986 of these genes were also differentially expressed between the subtypes in The Cancer Genome Atlas. The expression patterns of the most stereotypical Mesenchymal (CD44, Lyn, WT1, and BCL2A1) and Proneural (CD133, Olig2, Sox2, and Notch1) genes were confirmed by quantitative real-time PCR. Expression patterns also matched those of the original tumors similarly, if not identically, for each subtype after cell lines were established.[xiv]

2.2.1 Patient derived cell lines cultured as adherent cells

Patient GBM tumor cells were isolated[xv] and characterized into Proneural or Mesenchymal. Proneural Line 19 and Mesenchymal Line 83 were cultured in cell culture media (DMEM/F12, Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were cultured at 37°C, 5% CO_2 and fed 2-3 times per week. Passaging was done at 70% confluency and tests were completed on cells under passage 20.[xv] It was possible that mycoplasma infected both Line 19 and Line 83.

2.2.2 Patient derived cell lines cultured as neurospheres

Proneural Line 157 and Mesenchymal Line 326 were cultured in suspension as neurospheres. Neurospheres were suspended in cell culture media (DMEM/F12, Invitrogen) containing 1% B27 (Invitrogen), 1% glutamax (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen), 10 µg/mL heparin (Sigma-G-31149), 2 µg/mL basic fibroblast growth factor (Peprotech-100-18-B), and 2 µg/mL epidermal growth factor (Peprotech-AF-100-15). Heparin, bFGF and EGF were re-added to cell media twice a week. New media was made weekly. Cells were passaged twice weekly. Mesenchymal Line 326 cells were tested between passages 6-9 and Proneural Line 157 cells were tested between passages 2-3.

2.3 Analysis of single cell migration on PCL nanofibers using time lapse confocal imaging

Single cells were labeled with cell tracker dye (CMFDA, Green, Invitrogen) and seeded on sterilized nanofiber scaffolds at ~ 40,000 cells/well in a 12 well plate. After one hour, non-adherent cells were washed with single cell culture media and then placed in a weather controlled microscope stage (Precision Control LLC). Next, 50 µm z-stack

images were captured every 20 minutes for a total of 18 hours at a minimum of 3 random positions per well using an inverted microscope (Olympus IX 71). The images were made into migration movies and analyzed using the MTrack J plug in for Image J. Cells at were clustered or underwent proliferation were excluded.

2.4 Analysis of single cell adhesion on nanofibers

Fixed and sterilized scaffolds were incubated in single cell culture media for 1 hour. Single cells were labeled with cell tracker dye (CMFDA, Green, Invitrogen) and seeded on nanofiber scaffolds at ~ 40,000 cells/well in a 12 well plate. After thirty minutes, non-adherent cells were washed (3x) with single cell culture media and then placed in a weather controlled microscope stage (Precision Control LLC). Images (N = 3 per well) were taken using an inverted fluorescence microscope (Olympus IX 71) with a 10× objective. Cell adhesion was quantified for each subtype as average number of adhered cells per square millimeter ± SD.

2.5 Analysis of cell morphology on PCL nanofibers

2.5.1 Single cell morphology

Fixed and sterilized scaffolds were incubated in single cell culture media for 1 hour. Single cells were labeled with cell tracker dye (CMFDA, Green, Invitrogen) and seeded on nanofiber scaffolds at ~ 40,000 cells/well in a 12 well plate. Cells were placed in a weather controlled microscope stage (Precision Control LLC). After 24 hours, images (N = 3 per well) were taken using an inverted fluorescence microscope (Olympus IX 71) with a 10× objective. In order to quantify the elongation of cells on the fibers, feret diameter, the maximum distance in the elongated axis of the polarized single cell, was taken using ImageJ. The average ± SD feret diameter of each single cell type was reported in μm.

2.5.2 Neurosphere morphology

Fixed and sterilized scaffolds were coated in laminin (Sigma) for 1 hour at 37°C at a concentration of 50 µg/mL. Scaffolds were rinsed with Hank's Balanced Salt Solution (Sigma) (3x). Neurospheres were labeled with cell tracker dye (CMFDA, Green, Invitrogen) and seeded on nanofiber scaffolds at ~ 40,000 cells/well in a 12 well plate. Cells were placed in a weather controlled microscope stage (Precision Control LLC). After 24 hours, images (N = 3 per well) were taken using an inverted fluorescence microscope (Olympus IX 71) with a 10× objective. Vertical and horizontal feret diameters were taken with respect to nanofiber alignment using ImageJ (Figure 1). The ratio of elongation was calculated by dividing vertical by horizontal feret diameter. The average ± SD ratio of each neurosphere cell type was reported.

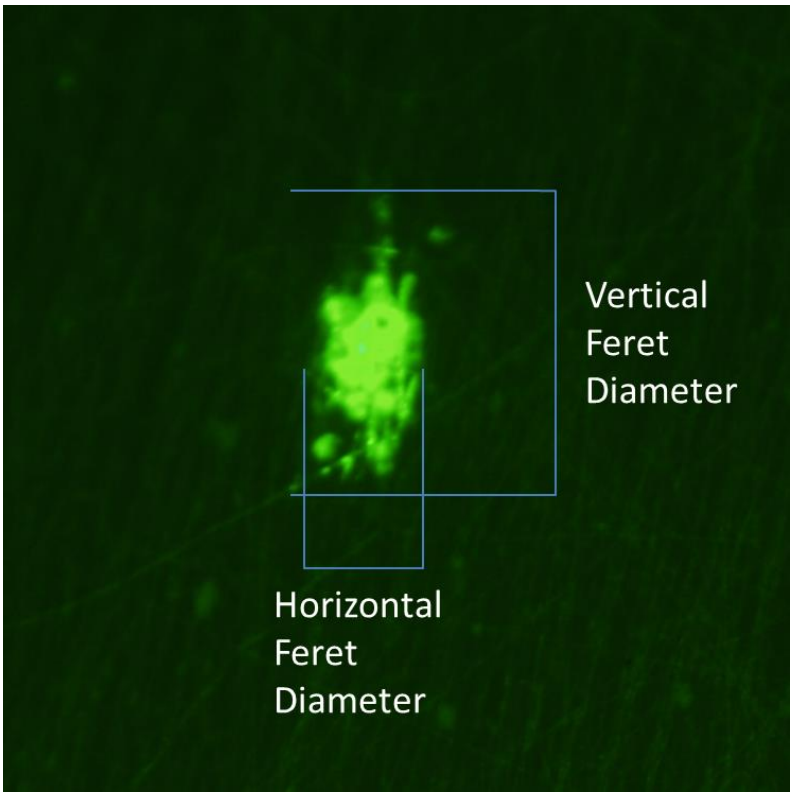

Figure 1: Vertical and horizontal feret diameters shown on elongated Pronerual Neurosphere adhered to laminin-coated PCL nanofibers. Distance measurements computed by ImageJ.

2.6 Image and Statistical Analysis

All data was analyzed using Microsoft Excel 2010. Statistical differences were calculated using the TTEST function. Data was considered to be a one sided distribution with unequal variance among cell lines. Unless otherwise noted, $p<0.05$ was considered to be statistically significant.

3. Results

3.1 Single Cell Migration on PCL nanofibers

Single cell migration speed was analyzed for the two subtypes on aligned PCL fibers using time-lapse confocal microscopy. Proneural Line 19 cells showed a significantly faster migration speed ($P<0.001$, 2 sample t-test) compared to Mesenchymal Line 83 cells. Plot of migration speed as a function of cell type is shown in Figure 2.

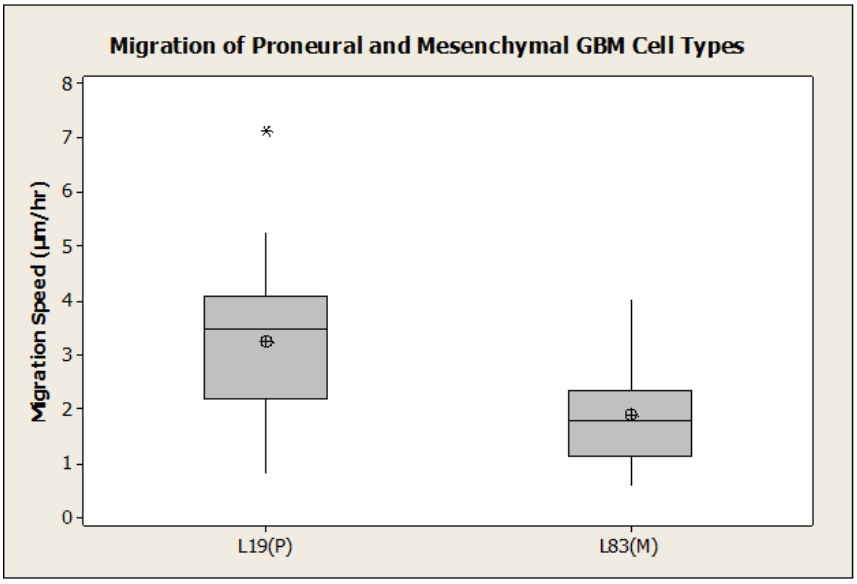

Figure 2: Migration speeds (µm/hr) for Pronerual (Line 19) and Mesenchymal (Line 83). $n_{L19} = 55$, $n_{L83} = 23$. * indicates statistically significant difference. Standard box-and-whisker notation used. ⊕ indicates mean.

3.2 Single Cell Adhesion on PCL nanofibers

Aligned nanofibers were used as an in vitro model to test how biomimetic topographies influenced Proneural and Mesenchymal attachment differently. Cells were only allowed to adhere for 30 minutes in order to avoid proliferating cells altering attached cell count. Figure 3 shows snapshots of the differences in adhesion between GBM subtypes.

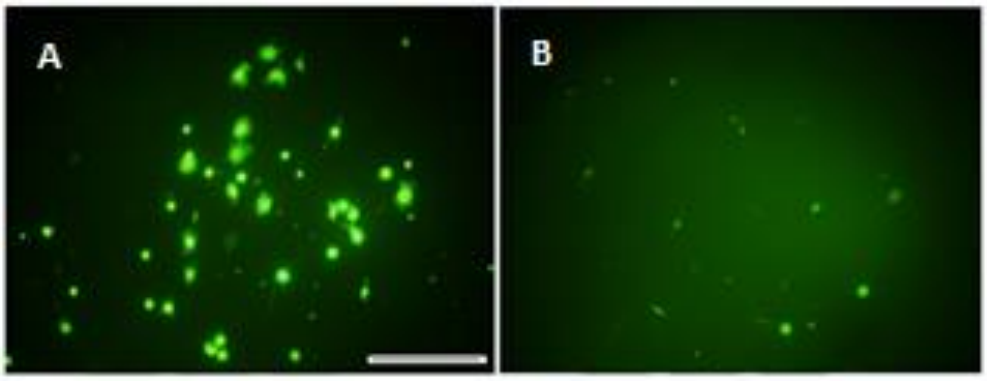

Figure 3: Single cell adhesion after non-adherent cells were rinsed at 30 minutes. (A) Proneural Line 19 (B) Mesenchymal Line 83. Scale bar indicates 200 μm.

Consistent with single cell migration, Proneural Line 19 showed increased affinity for nanofibers after 30 minutes (P=0.008, 2 sample t-test). Figure 4 shows the results of the single cell adhesion tests.

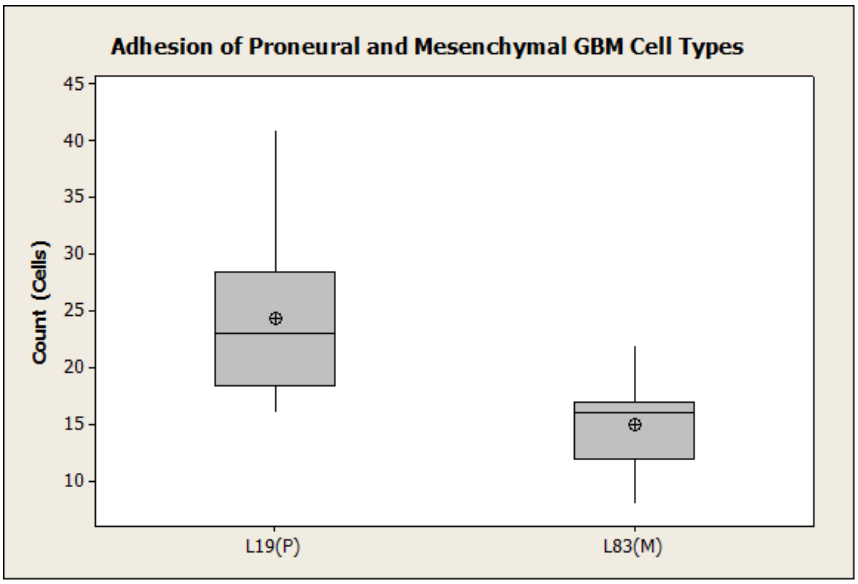

Figure 4: Adhesion as a function of single cell type. Proneural (Line 19) show greater initial attachment to nanofiber compared to Mesenchymal (Line 83). n_{L19} = 9, n_{L83} = 9. * indicates statistically significant difference. Standard box-and-whisker notation used. ⊕ indicates mean.

3.3 Morphology on PCL nanofibers

3.3.1 Single cell morphology

Consistent with adhesion and migration, the single cell morphological signatures of each of the tested GBM subtypes were distinct. Proneural Line 19 cells elongated much longer along the length of the polarized nanofibers, which is similar to the in vivo morphology of GBM (Figure 5).[xvi,xvii,xviii,xix]

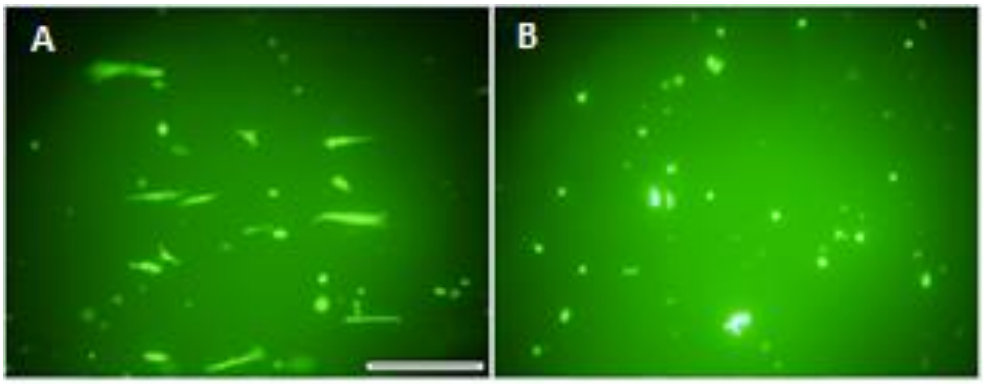

Figure 5: Cell morphology on nanofiber scaffolds. (A) Proneural Line 19 (B) Mesenchymal Line 83. Scale bar in (A) indicates 200 μm.

The extent of elongation was influenced by GBM subtype, though. Feret diameters are shown in Figure 6.

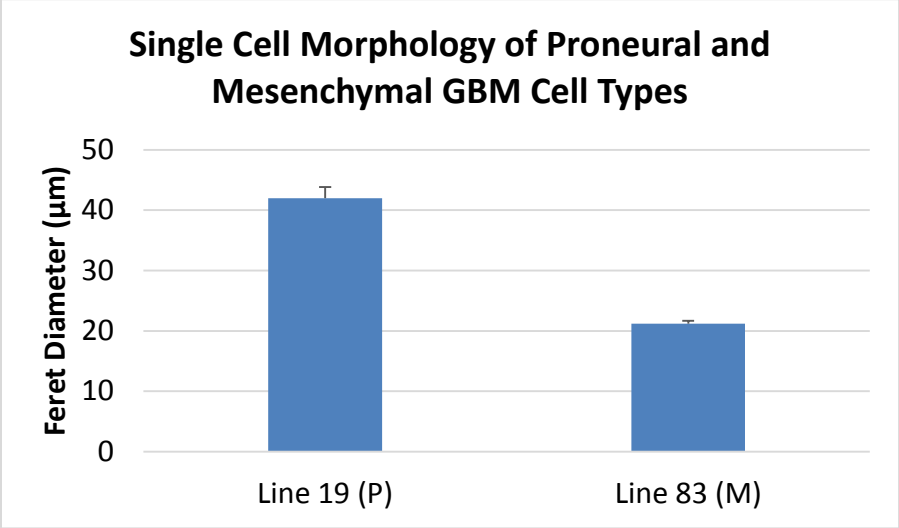

Figure 6: Feret diameter of single cell GBM subtypes on PCL nanofibers. $n_{L19} = 534$, $n_{L83} = 818$; 2-sample t-test: p-value < .001.

3.3.2 Neurosphere morphology

The morphological signatures of Proneural and Mesenchymal GBM neurospheres showed similar patterns to those cultured as single cells. Neurospheres were assumed to

be roughly spherical at initial seed time.

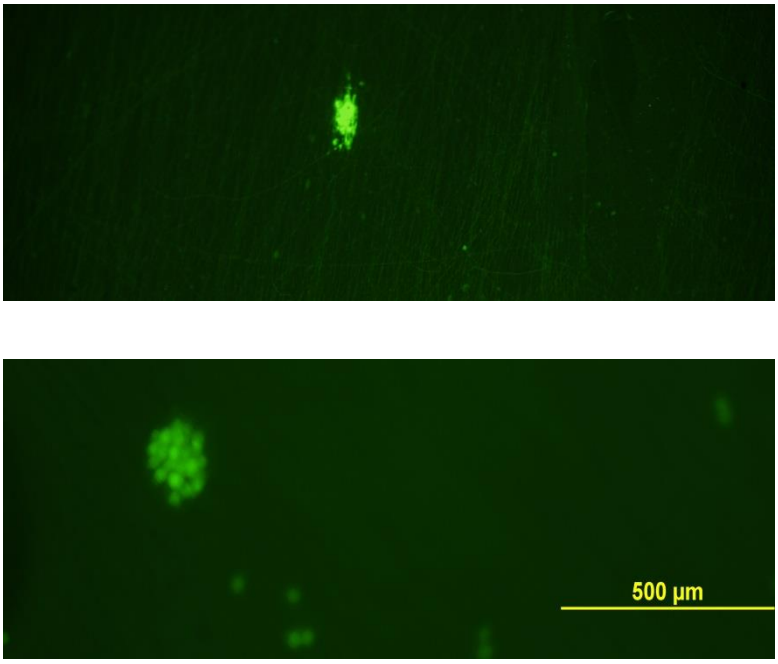

Figure 7: Neurosphere morphology on laminin-coated nanofiber scaffolds for (A) Proneural Line 157 and (B) Mesenchymal Line 326. Scale bar shown in (B).

Neurospheres did not show significant elongation on PCL scaffolds. Instead, nanofibers were coated with laminin, a commonly used extra-cellular matrix molecule which does not alter the characterization of either subtype. Upon seeding to laminin coated scaffolds, neurosphere elongation was significant in the Proneural subtype (P-value=0.0338, 2 sample t-test). Results are quantified by taking the ratio of vertical to horizontal feret diameter, relative to nanofiber alignment (Figure 8) and are shown in Figure 8.

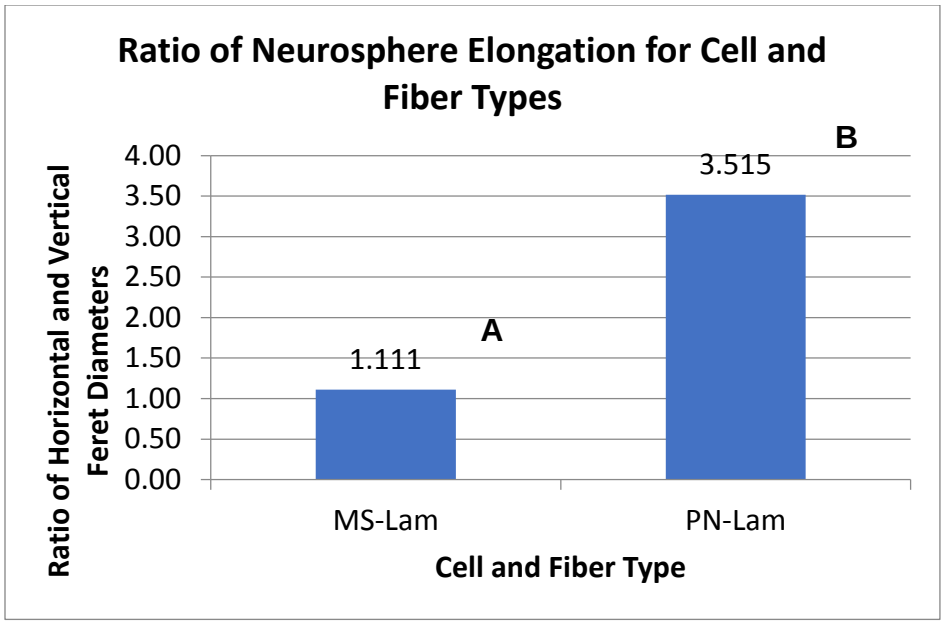

Figure 8: Feret diameter of GBM subtypes on PCL nanofibers coated with laminin. $n_{L157} = 4$, $n_{L326} = 9$; 2-sample t-test: p-value = 0.0339.

4. Discussion

Here, we demonstrate the phenotypic differences between Proneural and Mesenchymal subtypes of GBM, despite their similar treatments in the clinic. Highly aligned PCL electrospun nanofibers were used to mimic white matter tracts, one of the three major migratory highways for GBM. This model provides several advantages over traditional in vitro models used to test tumor cell migration, including its higher degrees of similarity in polarized topographical features, which allows for the more accurate quantification of tumor cell migration. The polarization of the fibers also is more biomimetic and allows for more accurate morphological testing. In addition, the mechanical modulus of PCL nanofibers is more representative of the myelin sheaths of white matter tracts, which have roughly the same order of magnitude of modulus.[xx] Electrospinning nanofibers allowed us to more accurately mimic white matter tracts so that adhesion, morphology and migration could accurately be tested in this in vitro study.

Adhesion of single cells on aligned PCL nanofiber scaffolds was compared for Proneural and Mesenchymal subtypes. Proneural cells showed significantly higher adhesion. This observation was in contrast to behavior of Proneural and Mesenchymal cells grown during culture periods. Proneural cells showed low proliferation and poor adhesion to cell culture plates throughout cell culture maintenance. Mesenchymal, on the other hand, exhibited high proliferation rates and adhesive behavior. This highlights the importance mechanical modulus and surface chemistry effects have on cell behavior for both

subtypes. Nevertheless, there were distinctions in adhesive behavior between Proneural and Mesenchymal subtypes.

Morphology of single cells on nanofiber scaffolds was also compared for Proneural and Mesenchymal subtypes. Proneural cells exhibited a significantly higher feret diameter compared to Mesenchymal. This behavior is consistent with adhesion testing results.

Single cell migration was compared for the two subtypes using time-lapse confocal microscopy. Once again, Proneural cells exhibited a significantly higher migratory potential, consistent with adhesion and morphology tests. Interestingly, these results challenge GBM behavior seen in the clinic, where Mesenchymal subtype is typically accepted as the most infiltrative of the subtypes.[xxi] These results could provide insight to the method of migration the subtypes use during invasion.

Neurosphere morphology was compared for the two subtypes on nanofiber scaffolds. Consistent with single cell morphology results, Proneural spheres elongated much more than Mesenchymal spheres. Visually, Proneural cells elongated and began to migrate out of the spheres, while cells in Mesenchymal spheres lost their polarity. This loss of polarity is analogous with the stereotypical loss in polarity for cells undergoing the EMT transition, observed in cancer progression and metastasis.[xxii,xxiii]

Morphology tests for neurospheres did not reveal significant differences on PCL fibers alone through our testing, although additional testing could provide the statistical power needed to obtain significant results. Tests for neurospheres were conducted on nanofiber scaffolds coated in laminin. Laminin is commonly used in neurosphere cell culture protocols and GBM subtype genetic expression has been shown to be conserved in its

presence.[xxiv] The drastic increase in elongation of Proneural neurospheres provides additional evidence of the sensitivity GBM subtypes have for surface chemistries.

Cell migration occurs when the cell body's shape is modified to interact with the surrounding ECM or, in this case, nanofiber scaffolds. The nanofibers serve as a substrate for migrating cells, where altering their cytoskeleton allows them to polarize and form membrane anchors, ultimately allowing for contraction and advancement. In vivo, cell-matrix and cell-cell interactions play a critical role in cell adhesion and migration. Laminin, for example, an ECM protein commonly found in the brain, stimulates migration out of glioma spheroids, possibly though increasing the expression of the receptor α3β1 integrin.[xxv,xxvi] Glioma cells have been shown not to migrate in serum-free media. The addition of an ECM-protein, though, typically induces migration.[xxvii] This behavior is even more prevalent in primary tumor spheroids,[xxviii] possibly explaining the drastic difference in morphological response amongst the neurospheres exposed to nanofiber scaffolds coated in laminin.

Other important molecules in cell migration are integrins and cadherins. Integrins are transmembrane glycoproteins that interact with ECM proteins and cell surface molecules. They connect to the cell's cytoskeleton and initiate focal adhesion sites.[xxix] Focal adhesion sites are more prevalent in cells that most often form and release adhesion sites.[xxx] Therefore, staining of integrins would show higher expressions among the more migratory subtype, presumably Proneural. NCAM, for example, decreases integrin expression[xxxi] and overexpression leads to a decrease in cell motility.[xxxii] NCAM expression would predictably be higher in Mesenchymal subtype.

Cadherins form calcium-dependent transmembrane, cell–cell adhesion complexes. They provide linkages to the intermediate filament network. Cadherins play a pivotal role in cell-cell adhesion and communication, and decreased function is associated with metastasis and poor-prognosis.[xxxiii] β-catenin, which links cadherins to the actin fibers, promotes cell-cycle progression. E-Cadherin, on the other hand, both suppresses β-catenin and cell movement. β-catenin would predictably show higher expression in Proneural subtype compared to Mesenchymal, while E-cadherin expression would be lower.

All in all, these results indicate a clear difference in phenotype between Proneural and Mesenchymal GMB subtypes. Electrospun nanofibers have previously been proven to closely mimic white matter tracts, a major migratory pathway for GBM, in the brain. These models were used to demonstrate clear differences in adhesion, morphology and migration for single cells and morphology for neurospheres, and these results pave the way for future investigation that would use this evidence to distinguish between clinical treatments for GBM patients with tumors of predominantly different subtypes.

5. Conclusions

Here, we compared the adhesive, morphological and migratory potentials of Proneural and Mesenchymal subtypes on highly aligned PCL electrospun nanofibers. This model allowed us to accurately mimic the mechanical modulus and topographical features of white matter tracts, a major migratory pathway for GBM, in an in vitro model. We demonstrated that there were clear differences in all tested parameters for both the subtypes in both single cell and neurosphere conditions. Migration differences correlated with differences in gene expression expected from cells based on subtype. Proneural cells, associated with increased expression of MAP2, a gene encoding for a microtubule-associated protein, showed increased migration compared to Mesenchymal, which exhibited low levels of NF1, a negative regulator of ras. These findings should have broad implications on future works concerning the migration of GBM subtypes. Migration studies conducted on GBM, both in vitro and in vivo, should take GBM subtype into account.

Ultimately, our group plans on conducting further studies in this domain. To gain additional support of differing adhesive properties between subtypes, adhesion proteins could be stained for by Western blotting. Proteins of interest include focal adhesion kinase (FAK), phospho-Tyr925 FAK (pFAK), [xi] β-catenin and E-cadherin. These proteins were chosen because of their association to focal adhesions, cell migration and adhesion. To confirm the relationship of migratory proteins, inhibitors to highly expressed proteins could be included in the model. For example, an FAK inhibitor (PF-228, PF-271, or

NVP-226)[xxxiv] could be used to nullify the difference in FAK expression between the subtypes and a sensitivity analysis could be done on the resulting effects FAK inhibition has on each subtype's migration. This could be repeated for several migratory protein inhibitors. This could provide insight that certain drugs are particularly effective or ineffective against one subtype but not the other. Known migration markers could then be stained for to detect which genes are the sources of the variations in migration. The same could be done for chemokines, in the place of anti-metastatic drugs. Data acquired from these future studies, in addition to our existing data, would ultimately lead to more effective care for GBM patients.

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